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Fabry disease due to D313Y and novel GLA mutations

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Manuscripts

1 Fabry disease due to D313Y and novel GLA mutations

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27 **Abstract**

28 **Objectives:** Our aim is to report five novel *GLA* mutations resulting in FD and provide
29 evidence of pathogenicity of the D313Y mutation regarding which contradictory data have
30 been presented in the literature.

31 **Setting and participants:** 25 family members of nine unrelated patients with definite FD
32 diagnosis, ten clinically suspected cases and eighteen members of their families were included
33 in this polycentric cohort study.

34 **Primary and secondary outcome measures:** Genotyping and measurement of lyso-Gb₃ was
35 performed in all individuals. The α -Gal A activity was measured in all males as well as
36 plasma and urine Gb₃ concentration in selected cases. Optical and electron microscopy was
37 performed in kidney biopsies of selected patients. All the above were evaluated in parallel
38 with the clinical data of the patients.

39 **Results:** Sixteen new cases of FD were recognised, three of which were carrying already
40 described *GLA* mutations. Five novel *GLA* mutations, namely c.835C>T, c.280T>A,
41 c.924A>C, c.511G>A and c.453C>G, resulting in a classical FD phenotype were identified.
42 Moreover, FD was definitely diagnosed in five patients carrying the D313Y mutation. Eight
43 D313Y carriers were presenting sings of FD despite not fulfilling the criteria of the disease,
44 two had no FD signs and two others were apparently healthy.

45 **Conclusions:** Five novel *GLA* pathogenic mutations are reported and evidence of
46 pathogenicity of the D313Y mutation is provided. It seems that the D313Y mutation is
47 related with a later-onset milder than the typical phenotype with normal lysoGb₃
48 concentration. Our study underlines the significance of family members genotyping and
49 newborn screening in avoiding misdiagnoses and crucial delays of diagnosis and treatment of
50 the disease.

Strengths and limitations of this study

- Novel GLA mutations resulting to a classical Fabry disease phenotype are presented.
- The clinical impact of the D313Y mutation of the GLA mutation is analysed in a significant number of male and female carriers at various ages.
- This study offers strong evidence that the D313Y mutation could be pathogenic, indicating that therapy should be considered when appropriate.
- The main limitation is the lack of detailed clinical data in older participants.

INTRODUCTION

FD or Anderson-Fabry disease is an X-linked inherited metabolic disorder, that results from mutations in the α -gal A gene (*GLA* gene), leading to reduction of the enzyme activity and subsequent accumulation of Gb₃ (or GL-3) in plasma, urine and cellular lysosomes throughout the body. These depositions cause a multisystemic pathology with life-threatening manifestations, including renal failure, cardiac and cerebrovascular disease [1, 2].

More than 600 currently known *GLA* mutations have been identified [3, 4], as causing a variety of clinical manifestations. Most of them are unique to a family (private) and therefore genotype-phenotype correlation is limited [5]. Diagnosing FD is challenging due to the range of disorders that mimic the disease and the great variety of atypical clinical presentations. As a result underdiagnosis and misdiagnosis of FD lead to late diagnosis, delays in appropriate treatment and a subsequent negative prognosis [6]. Human genetic analysis must be performed, in order to exclude or verify a mutation of the *GLA* [7]. Once a diagnosis has been made, biochemical and molecular genetic analysis, as well as genetic counselling, should be made available to all family members [8]. A detailed pedigree analysis for each patient presenting with FD is crucial [9], as it can inform the diagnosis of the proband and the identification of all at-risk relatives [10].

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75 Our aim is to report five novel *GLA* mutations resulting in FD and provide evidence of
76 pathogenicity of the D313Y mutation regarding which contradictory data have been presented
77 in the literature.

78 **METHODS**

79 **Study population**

80 A cohort of 62 subjects was involved in the study selected between families of 9
81 unrelated patients with definite diagnosis of FD as well as amongst cases with nephrological,
82 cardiac or neurological symptoms suspicious of this diagnosis. Eighteen family members of
83 the last cases were also examined after the confirmation of diagnosis. The presence of the
84 D313Y mutation in Greek population was examined by genotyping of 145 apparently healthy
85 subjects (70 males, 75 females).

86 Written informed consent was obtained from each subject or an accompanying relative,
87 where legally appropriate. The study was approved by the institutional review board of the
88 University of Thessaly, Larissa.

89 **Clinical assessment**

90 Patients' medical records were revaluated and a detailed medical history of the family
91 members was obtained especially in regard with heart or kidney disease, cerebrovascular
92 events, death at young age and respective causes of death. All study participants
93 underwent physical examination particularly focused on cardiac, renal and neurological signs
94 and symptoms. A detailed pedigree was constructed for every family and newborn screening
95 was performed once.

96 **Laboratory evaluation**

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3 97 In DBS, we measured α -Gal A activity in all male subjects by tandem mass
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5 98 spectrometry, lyso-Gb₃ in all subjects by HPLC and tandem spectrometry [11] as well as
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7 99 plasma and urine Gb₃ concentration in selected cases by tandem mass spectrometry.
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10 Optical and electron microscopy was performed for the study of kidney biopsies
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14 102 **Genotyping**

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17 103 Analysis of primary data was conducted with Ion Reporter software v.5.2 (Thermo
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19 104 Scientific). Common polymorphisms (UCSC Common SNPs) were excluded and
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21 105 pathogenicity of variations was predicted by bioinformatic analysis using PhyloP, SIFT,
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23 106 Grantham and PolyPhen tools, in comparison to their global (1000 Genomes Global Minor
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25 107 Allele Frequency, ExAC) and European frequency (5000 Exomes European Minor Allele
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27 108 Frequency). The characterization of variants was based on the recommendations of the
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29 109 American College of Medical Genetics and Genomics (ACMG) and the Association for
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31 110 Molecular Pathology [12]. Novel mutations were verified by standard Sanger sequencing
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33 111 using Variant Reporter software v1.1 (Applied Biosystems).
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37 112 **RESULTS**

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40 113 Six (all with definite FD) out of the 62 genotyped subjects were carrying four
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42 114 previously described *GLA* mutations: c.334C>T (p.Arg112Cys, R112C), c.644A>G
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44 115 (p.Asn215Ser, N215S), c.1153A>C (p.Thr385Pro, T385P) and c.782G>T (p.Gly261Val,
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46 116 G261V).
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50 117 The c.937G>T (p.Asp313Tyr, D313Y, NM_000169.2) mutation was revealed in
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52 118 seventeen individuals (54±14, range 27– 78 years), seven males (61±11, range 45– 78 years)
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54 119 and ten females (49±15, range 27– 70 years) but in none of the healthy subjects. Patients'
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56 120 clinical and laboratory findings are presented in Table 1.
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The diagnosis of FD was definitely posed according to the recently published criteria of the disease [13], in five carriers of this mutation (54±9, range 45– 65 years). The first of them (patient no.1, Table 1), a 52-year-old man was initially diagnosed at the age of 48 with CKD stage III and was not presenting cardiac or other FD signs. Kidney biopsy performed because of non-nephrotic proteinuria, microscopic haematuria and raised serum creatinine, revealed focal and segmental glomerulosclerosis (FSGS) – collapsing variant. At that time enzyme activity and plasma lyso-Gb₃ concentration were normal. Three years after initial presentation the patient was suffering end stage renal disease and extreme acroparesthesias. Revaluation of kidney biopsy by higher magnification uncovered focal cytoplasmic microvacuolization of enlarged podocytes (Fig.1) while a decreased by 50% α-gal A activity and an increased plasma (7.52 nmol/mL, reference: 0.8-4.52) and urine Gb₃ concentration (147.49 nmol/g, reference: <29.00) were detected. The patient commenced dialysis and ERT with rapid clinical improvement.

A second carrier of the D313Y mutation was a 46-year-old female (patient no. 4, Table 1) who had suffered a TIA and two ischaemic strokes that had been considered of unknown origin. On revaluation, one year after the last stroke the patient appeared with microalbuminuria, oedema and acroparesthesias on both hands. The microalbuminuria was duplicated after three months and acroparesthesias worsened. Gb₃ concentration was pathological in urine (54.08 nmol/g) but normal in plasma. LysoGb₃ concentration was normal at that time and remained stable for one more year.

The mother of the above patient (patient no. 5, Table 1), 65 years old, had been diagnosed at the age of 50 with MS and was receiving relative medication, without clinical benefit. She was also receiving treatment for pain in extremities that were attributed to RA. During the recent years the patient suffered mobility impairment (reported as spastic quadriplegia after a neurological examination), depression and dementia. The evaluation of

the patient revealed pathological plasma Gb₃ concentration (4.7 nmol/mL), WML on brain MRI and normal kidney function. LysoGb₃ concentration was normal and remained so during a 6-month-follow up.

Another carrier of the D313Y mutation was a 45-year-old male (patient no.7, Table 1) on dialysis due to CKD by the age of 25. No kidney biopsy was performed at that time. He reports episodes of haematuria during childhood and adolescence, attributed at that time to vesicoureteral reflux. Enzyme activity was slightly decreased (2.4 µmol/l/h, reference: ≥2.6) and plasma lyso-Gb₃ concentration was normal. Brain MRI revealed WML and vertebrobasilar vessel changes. Moreover, increased echogenicity of cardiac interventricular septum on the echocardiogram and sensorineural hearing loss of higher frequencies. The patient commenced ERT.

The last patient with D313Y mutation definitely diagnosed as suffering FD was a 60-year-old female (patient no.17, Table 1) who was presenting cornea verticillata corneopathy, WML and ischaemic infarcts on brain MRI despite that no stroke is reported, acroparesthesias and GI symptoms (pain-diarrhoea) since adolescence, hypohidrosis, hearing loss and LVH.

Among the remaining twelve D313Y mutation carriers there were two patients with no FD signs and eight patients (55±17, range 27–78 years) presenting other FD signs mainly neurological that, however, can not document a definite diagnosis of FD. Especially patient no.6, Table 1 (62-year-old female) suffers acroparesthesias and GI symptoms since adolescence and presents with lysoGb₃ at 1.7 ng/ml (reference: ≥ 1.8). Accordingly patient no.16, Table 1 (64-year-old male) presents with CKD, diabetes mellitus and hearing loss, while on brain MRI presents multiple ischaemic infarcts despite that no stroke is reported. The remaining two D313Y mutation carriers are apparently healthy.

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Novel *GLA* mutations (5) (Fig. 2) were detected in sixteen members (42±19, range 1–73 years) of five unrelated families (Table 2), all fulfilling the diagnostic criteria of a definite diagnosis of FD [13]. The c.835C>T mutation (p.Gln279Ter, Q279X) in exon 6 of the *GLA* gene was identified in four members (30±24, range 1– 60 years) of a Greek family. The proband, a 31-year-old male, was presented at the age of 23 with proteinuria (3.5 gr/24h), microscopic haematuria, slightly deteriorated kidney function, right bundle brunch block, mild hypertension and angiokeratomas in the arms and the loin area. He reported pain in the extremities especially during infections and inability to sweat. The α-gal A activity was close to zero. Cardiac MRI showed moderate LVH. The kidney biopsy showed cytoplasmic vacuolization and extended lysosomal accumulations in all types of kidney cells, especially in the podocytes (Fig. 3). The patient is currently under ERT with beneficial results in regard with kidney function, proteinuria and pain. Three other members of the family were revealed having with the same mutation and all presented clinical signs of FD. The proband’s mother (60 years old) reported a possible TIA at the age of 32. Kidney examination showed albuminuria (> 500mg/24h), microscopic haematuria and normal kidney function. Cardiac MRI revealed severe LVH (cardiac interventricular septum over 19mm) and on skin examination she showed angiokeratomas in the arms. Her mother (the proband’s grandmother) had died at the age of 62, due to cardiac arrest. She suffered from severe LVH and acroparesthesias, which at that time were attributed to Raynaud’s phenomenon. The proband’s sister (27-year-old) has elevated lyso-Gb₃ concentration (2.7 ng/ml) and screening of her newborn daughter revealed the mutation too.

Four patients (46±11, range 30– 56 years) belonging to another Greek family were carrying the c.280T>A (p.Cys94Ser, C94S) mutation. The proband, a 48-year-old female, was diagnosed with FD a year before after presenting increased lyso-Gb₃ concentration (5.4 ng/ml), increased plasma Gb₃ concentration (6.14 nmol/ml), microalbuminuria of no other

194 origin, cornea verticillata corneopathy, acroparesthesias in both hands and dyshidrosis.

195 Clinical and laboratory data of the other suffering members of the family are presented in
196 Table 2.

197 The c.924A>C mutation (K308N - p.Lys308Asn) in exon 6 of the *GLA* gene was
198 identified in three members (44±26, range 17– 69 years) of a Greek family. The proband, a
199 46-year-old male, was presented at the age of 34 with albuminuria (0,5-0,6 gr/24h),
200 microscopic haematuria since ten years and slight hypertension. The kidney function was
201 normal. Kidney biopsy showed slight mesangial proliferative damages and cytoplasmic
202 microvacuolization of podocytes. The α -gal A activity was almost zero. After 10-year-
203 follow up he suffered of LVH and proteinuria (1.8 gr/24h). The patient is currently under
204 ERT. His mother and daughter carry the same mutation. The probands grandmother had
205 died at the age of 68, suffering of severe LVH and end-stage heart failure.

206 The c.511G>A mutation (G171S - p.Gly171Ser) in exon 3 of the *GLA* gene was
207 identified in two members (37 ± 4, range 34– 39 years) of an Albanian family living in
208 Greece. The proband, a 39-year-old male, was diagnosed at the age of 32 with severely
209 deteriorated kidney function and proteinuria. No biopsy was performed at that time, due to the
210 small size of the kidneys. After nearly a year he presented with severe clinical and laboratory
211 findings of acute renal failure and need of dialysis. Normal kidney function was never
212 restored. FD was definitely diagnosed at the age of 37, as the α -gal A activity was extremely
213 low and lyso-Gb₃ concentration was 11.9 ng/ml. The patient is suffering of LVH, increased
214 pulmonary artery diameter, dilatation of the ascending aorta and aortic valve stenosis, because
215 of which he underwent a valve replacement surgery. Ophthalmological evaluation indicated
216 lipid deposition with blurriness of the cornea. The patient is currently under ERT. His 34-
217 year-old brother was identified with the same mutation and has extremely low α -gal A
218 activity, lyso-Gb₃ concentration 12.9 ng/ml and albuminuria of no other aetiology.

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219 Lastly the c.453C>G mutation (Y151X - p.Tyr151Ter) in exon 3 of the *GLA* gene was
220 revealed in three members (55 ± 16 , range 42– 73 years) of an other Greek family. The
221 proband, a 50-year-old male, was initially misdiagnosed at the age of 36 with SLE, as he
222 suffered from small joints arthralgia both in hands and feet, low-grade fever and positive
223 antinuclear antibodies (ANA). A kidney biopsy performed at the time due to proteinuria (2.4
224 gr/24h) and deteriorated kidney function, indicated a possible SLE nephritis (class III –
225 WHO). He submitted on treatment for SLE for some years without clinical benefit. However
226 angiokeratomas in the pelvic area with which the patient was presented were progressing over
227 the time. This finding necessitated the revaluation of the patient. Kidney biopsy showed
228 cytoplasmic microvacuolization of enlarged podocytes, as well as segmental sclerosis in
229 some glomeruli. The α -gal A activity was found pathological low (0.06 $\mu\text{mol/l/h}$). SLE
230 treatment stopped and the patient commenced ERT. At that time the patient presented pain
231 crisis, acroparesthesias, hypohidrosis, temperature intolerance, skin lesions (angiokeratomas),
232 cornea verticillata corneopathy, nephropathy and mitral valve prolapse / insufficiency. The
233 proband's mother (73 years old) presented at the age of 64 with multiple parapelvic kidney
234 cysts, nephropathy, mitral valve prolapse, rhythm abnormalities, LVH and WML on brain
235 MRI. She was also suffering of chronic cough and depression. The probands sister was
236 misdiagnosed too, as years ago she was reporting severe joint pains attributed to mixed
237 connective tissue disease (MCTD). After the probands diagnosis and at the age of 41 she
238 genotyped and, at that time, the *GLA* mutation was found. Thereafter, she presented slight
239 albuminuria, mitral valve prolapse, mitral and aortic valve insufficiency. Autonomic and
240 central nervous system where also affected, as the patient reported acroparesthesias,
241 temperature intolerance and tinnitus. All three patients were diagnosed with cornea verticillata
242 corneopathy and are currently under ERT.

243 **DISCUSSION**

244 The D313Y mutation

245 Contradicting results about the pathogenicity of this mutation have been reported in the
246 literature since its first description on 1993 [14]. The mutation has been detected in many
247 series of patients presenting signs of FD [15, 16, 17, 18, 19, 20, 21, 22]. However, Niemann
248 et al. [23] describes this variant as non pathogenic, although his two patients were presenting
249 decreased α -gal A activity. Similarly, Oder et al. [24] supports that the D313Y genotype does
250 not lead to severe organ manifestations as seen in genotypes known to be causal for classical
251 FD and Froissart et al. [25] characterizes the mutation as “pseudodeficient allele” implying
252 that it is a sequence variant which encodes an enzyme that is transported to the lysosomes,
253 where it has about 75% of normal enzymatic activity. The D313Y mutation has been also
254 referred as polymorphism [17], despite that, according to the ExAC and 1000 Genomes
255 databases, its frequency in the World and European population is below 1%. Finally, it must
256 be mentioned that by bioinformatics analysis this mutation is predicted as probably damaging
257 (PolyPhen-2) or damaging (SIFT).

258 In our study five of seventeen carriers of the D313Y mutation (54 ± 9 , range 45– 65
259 years) proved as suffering definite FD according to the recently published criteria of the
260 disease [13]. The presentation of the disease in our patients indicates that the mutation results
261 in a milder phenotype, with later onset of symptoms. This phenotype, also including milder
262 mono- or oligosymptomatic cases [17], is characterised as atypical or type 2 [26]. The late
263 onset of clinical symptoms and the milder than the typical phenotype of FD in these patients
264 can be partly explained by the high α -gal A residual activity, since there is evidence that the
265 mutated α -gal A reaches intracellularly the lysosomes [27]. Actually, in nearly all male
266 D313Y carriers of our study (61 ± 11 , range 45– 78 years) α -gal A activity was decreased in a
267 range of 56.2-87.5% comparing to normal. Another reason for the decreased activity of the
268 D313Y enzyme in plasma could be a functional intolerance to blood plasma neutral pH

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conditions. This effect is irreversible and, once in contact with a neutral or basic pH environment D313Y enzyme remains inactive, even if transferred to optimal pH [19, 27].

Interestingly, the vast majority of our D313Y patients were presenting with neurological symptoms and signs. Moreover, one of them was misdiagnosed as MS while two carriers of the mutation, in whom definite FD diagnosis has not been established as yet, are diagnosed and treated as MS. Similarly the detailed study of the renal biopsy in one of our cases underlies the significance of early detection of Fabry specific findings in cases with FSGS.

Novel mutations

The pathogenicity of the above mentioned novel mutations has been undoubtedly proved as all carriers suffer definite FD. Of them the c.835C>T (p.Gln279Ter, Q279X) is a nonsense mutation causing an interruption of the reading frame by a premature stop codon, which results in a truncated protein. A truncated protein and the subsequent loss of its functionality is strong evidence that the mutation is probably pathogenic for FD [12]. No amino acid change at this position has ever been described in NCBI and Fabry Database (<http://fabry-database.org/>) [28]. Accordingly the c.453C>G (p.Tyr151Ter, Y151X) mutation causes an interruption of the reading frame by a premature stop codon, which results in a truncated protein and subsequent loss of its functionality. This is a strong evidence that the mutation could be pathogenic for FD [12]. A further evidence that this mutation is pathogenic is provided by the fact that the same amino acid change at this position but in a different nucleotide has already been described as disease-causing [29, 17].

As far as the c.280T>A (C94S), c.924A>C (K308N), c.511G>A (G171S) mutations are considered, bioinformatics analysis supports their disease causing effect as they are predicted as probable damaging (PolyPhen-2) and/or damaging (SIFT). A further evidence of their pathogenicity could be the fact that the same aminoacid changes at the corresponding

positions but in different nucleotides have already been described as disease causing [30, 31, 29,].

CONCLUSIONS

We report five novel *GLA* mutations causing FD. Moreover, we offer strong evidence that the D313Y mutation could be pathogenic. It seems that this mutation is related with a later-onset milder than the typical phenotype with normal lysoGb₃ concentration.

Additionally our study underlines the significance of family members genotyping, newborn screening and genetic counselling in avoiding misdiagnoses and crucial delays of diagnosis and treatment of the disease. Finally we confirmed the fact that heterozygous females may develop mild to severe FD as well as that genotype-phenotype correlation does not exist even among the members of the same families.

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305 **Table 1.** Characteristics of patients carrying the D313Y mutation.

Patient / Gender / Age (years)	FD related clinical findings	Previous diagnosis
1 / M / 52	End stage renal disease – dialysis, acroparesthesias, renal cysts, elevated plasma and urine Gb ₃ concentration	FSGS nephropathy
2 / F / 30	Healthy	no
3 / F / 70	Healthy	no
4 / F / 46	Three strokes of unknown origin, WML on brain MRI, micro-albuminuria, oedema, acroparesthesias, elevated urine Gb ₃ concentration	no
5 / F / 65	Multiple WML on brain MRI, depression, elevated plasma Gb ₃ concentration	MS, RA, spastic quadriplegia
6 / F / 62	Acroparesthesias, GI symptoms since adolescence	no
7 / M / 45	End stage renal disease on dialysis, WML and vertebrobasilar vessel changes on brain MRI, increased cardiac interventricular septum echogenicity, hearing loss of higher frequencies	Nephropathy of unknown origin
8 / M / 62	End stage renal disease on dialysis	no
9 / F / 36	WML on brain MRI, acroparesthesias	MS
10 / M / 78	End stage renal disease on dialysis	RA
11 / F / 47	LVH, CKD	no
12 / M / 68	No FD signs	Myopathy
13 / F / 46	No FD signs	NMO
14 / F / 27	WML on brain MRI	MS
15 / M / 61	End stage renal disease on dialysis	Diabetic nephropathy
16 / M / 63	CKD, hearing loss, multiple ischaemic infarcts on brain MRI	no
17 / F / 59	Cornea verticillata, hearing loss, LVH, acroparesthesias, GI symptoms (pain – diarrhoea) since adolescence, hypohidrosis, T2 -WML or ischaemic infarcts on brain MRI	no

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Table 2. Clinical characteristics of patients with the five novel mutations.

Mutation (NM_000169.2)	Clinical data
c.835C>T p.Gln279Ter Q279X	CKD, haematuria, proteinuria, pain in extremities, dyshidrosis, angiokeratomas, zero activity of a-gal A, LVH, kidney biopsy consistent with FD, TIA, pathological elevated lyso-Gb ₃
c.280T>A p.Cys94Ser C94S	Microalbuminuria, end stage renal disease – dialysis, cornea verticillata corneopathy, acroparesthesias, dyshidrosis, LVH, WML on brain MRI, zero activity of a-gal A, pathological elevated lyso-Gb ₃ , pathological elevated Gb ₃ in plasma/urine
c.924A>C p.Lys308Asn K308N	CKD, haematuria, kidney biopsy findings related to FD, zero activity of a-gal A, LVH
c.511G>A p.Gly171Ser G171S	Proteinuria, end stage renal disease – dialysis, LVH, valvulopathy, cornea verticillata corneopathy, extremely low activity of a-gal A, pathological elevated lyso-Gb ₃
c.453C>G p.Tyr151Ter Y151X	Proteinuria, end stage renal disease – dialysis, kidney biopsy findings related to FD, pain in extremities – acroparesthesias, severe angiokeratomas, hypohidrosis – temperature intolerance, cornea verticillata corneopathy, LVH, valvulopathy, rhythm abnormalities, WML on brain MRI, tinnitus, low activity of a-gal A

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Figure 1. Optical microscopy findings of renal biopsy from a male FD patient carrying the D313Y mutation of the *GLA*. (a) Glomerulus with segmental sclerosis - PAS X 400. (b) Segmental sclerosis with features of the “collapsing” variant - Jones’ silver X 400. (c) Pale appearing glomerulus with a small area of sclerosis adhering to Bowman’ s capsule - PAS X 400. (d) Cytoplasmic microvacuolization of podocytes, suggestive of FD - PAS X 400.

Figure 2. Sanger confirmation of novel mutations

Figure 3. Electron microscopy findings of renal biopsy from a male FD patient carrying the Q279X mutation. Multi-lamellated myelin figures (“zebra” bodies), typical finding of FD, are marked with black arrows in (a) methylene blue semithin section, (b) tubular cells and a fibroblast and (c,d) podocytes.

Abbreviations

α -gal A: Enzyme α -galactosidase A; ACMG: American College of Medical Genetics and Genomics; ANA: Antinuclear antibodies; CKD: Chronic kidney disease; DBS: Dried blood spot; ERT: Enzyme replacement therapy; FD: Fabry disease; FSGS: focal and segmental glomerulosclerosis; GI: Gastrointestinal; GLA: α -galactosidase A gene; GL-3 or Gb3: globotriaosylceramide; LVH: Left ventricular hypertrophy; lyso-Gb3: globotriaosylsphingosine; MRI: Magnetic resonance imaging; MV: mean value; MS: Multiple sclerosis; NMO: Neuromyelitis optica; RA: Rheumatoid arthritis; SD: Standard deviation; SLE: Systemic lupus erythematosus; TIA: Transient ischemic attack; UT: Under treatment; VUS: Variant of uncertain significance; WML: White matter lesions;

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Availability of data and materials

All data supporting our findings are included in the manuscript.

Authors' contributions

KK: collection, analysis and interpretation of the data, pedigree analysis, literature review, drafting and revision of the manuscript. KS: Renal biopsy and analysis on electron microscope. PP: Renal biopsy and analysis on optical microscope. MS, MZ and GL: genotyping and bioinformatics analysis. KS, PP, EM, PK, CK, AO, JK: treating physicians of patients. AEG: design and coordination of the study, revision of the manuscript. All authors approved the final version of the manuscript.

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Competing interests

KK received travel assistance from Shire and Genzyme and speaker’ s honoraria from Shire. KS received travel assistance and speaker’s honoraria from Shire. PP and AO received travel assistance from Genzyme and Shire. CK received travel assistance from Shire. AEG received research grants from Shire. The authors MZ, GL, MS, EM, PK, JK, report no competing interests.

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Consent for publication

Written informed consents for this publication were obtained from patients. A copy of the consent form is available for review by the Editor of this journal.

Ethics approval and consent to participate

This report was approved by the institutional review board of the University of Thessaly, Larissa. Written informed consents for participation were obtained from patients.

361 **References**

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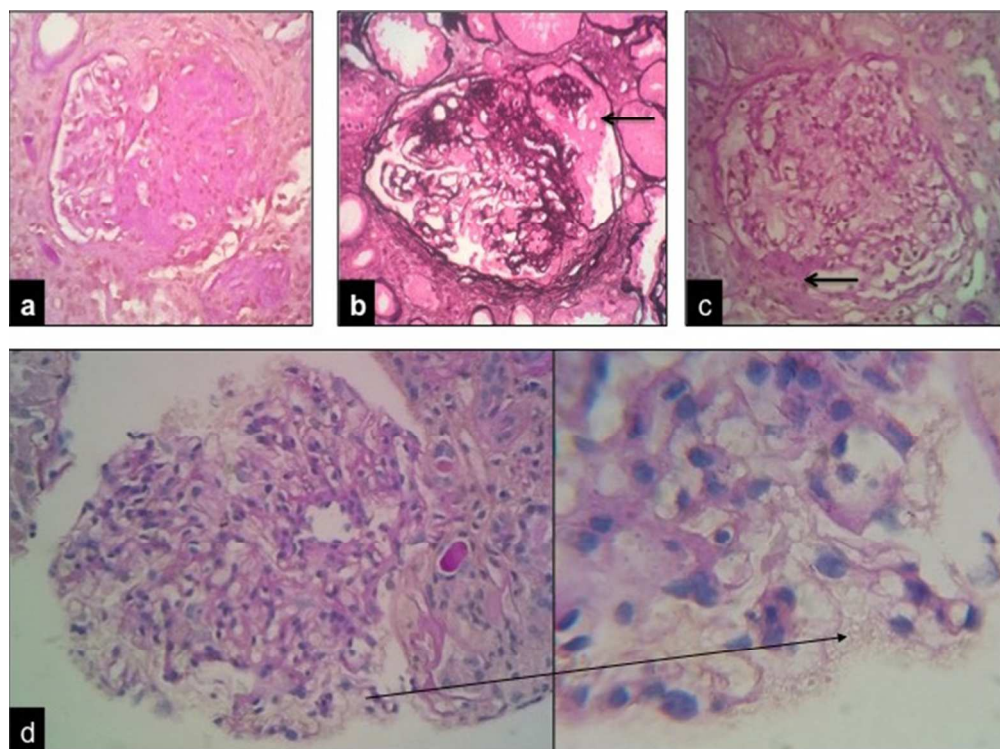


Figure 1. Optical microscopy findings of renal biopsy from a male FD patient carrying the D313Y mutation of the GLA. (a) Glomerulus with segmental sclerosis - PAS X 400. (b) Segmental sclerosis with features of the "collapsing" variant - Jones' silver X 400. (c) Pale appearing glomerulus with a small area of sclerosis adhering to Bowman's capsule - PAS X 400. (d) Cytoplasmic microvacuolization of podocytes, suggestive of FD - PAS X 400.

224x167mm (72 x 72 DPI)

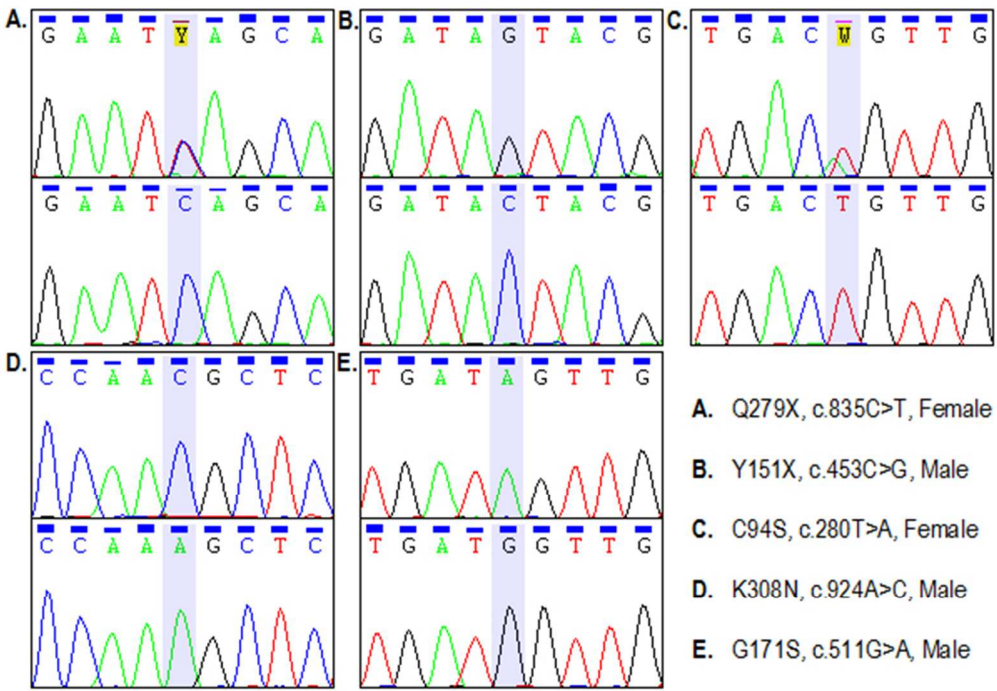


Figure 2. Sanger confirmation of novel mutations.

158x112mm (96 x 96 DPI)

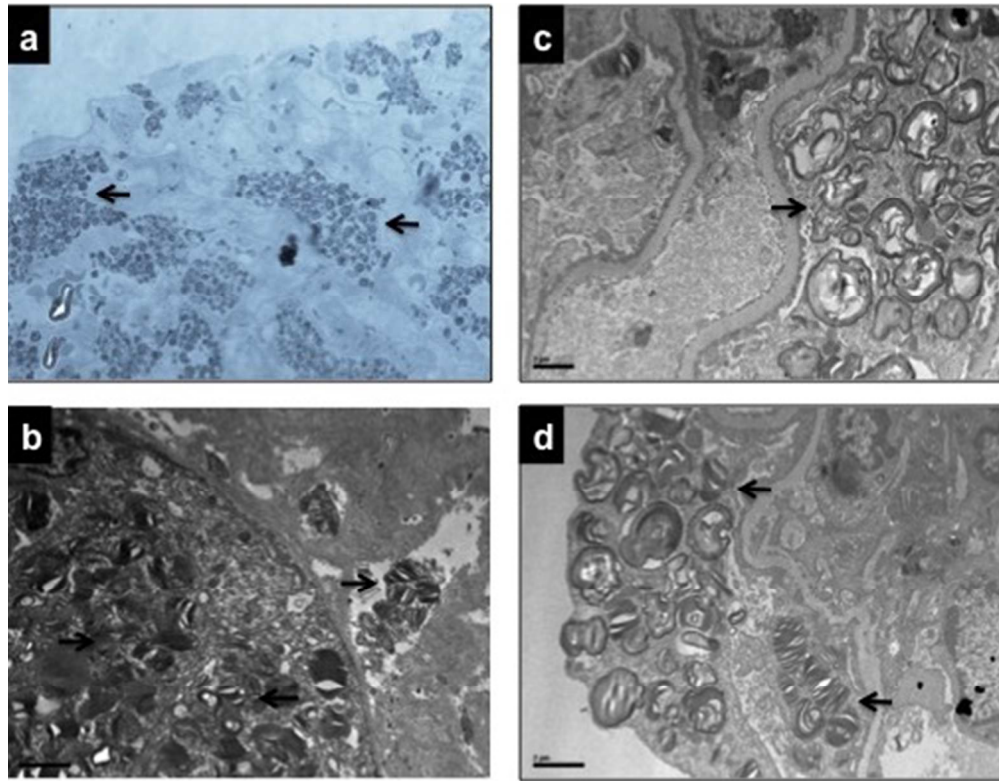


Figure 3. Electron microscopy findings of renal biopsy from a male FD patient carrying the Q279X mutation. Multi-lamellated myelin figures ("zebra" bodies), typical finding of FD, are marked with black arrows in (a) methylene blue semithin section, (b) tubular cells and a fibroblast and (c,d) podocytes.

178x139mm (72 x 72 DPI)

Koulousios et al.
STROBE Research Checklist

	Item No	
Title and abstract	1	Title [Page 1]: Fabry disease due to D313Y and novel GLA mutations Abstract [Page 3] The study is a polycentric observational cohort study which addresses the question of pathogenicity of specific Fabry disease mutations, using cohort data. Abstract [Page 3]: The abstract summarises the objectives, settings and participants, primary and secondary outcome measures, results and conclusions of the study.
Introduction		
Background/rationale	2	Introduction [Page 4] Introduction to the scientific background of Fabry disease and <i>GLA</i> mutations.
Objectives	3	Introduction [Page 5] Aim of the study is to report five novel <i>GLA</i> mutations resulting in FD and provide evidence of pathogenicity of the D313Y mutation regarding which contradictory data have been presented in the literature.
Methods		
Study design	4	Methods - Study population [Page 5] - Polycentric observational cohort study, multiple patient cases from various medical units all over Greece (polycentric study). - The study was approved by the institutional review board of the University of Thessaly, Larissa.
Setting	5	Methods - Study population and Clinical assessment [Page 5] - Patient cases from multiple medical units in various locations all over Greece. - Family members of the majority of the Greek FD patients, who were diagnosed during the last years. - Pedigree analysis after personal interviews with the selected individuals in their places of residence.
Participants	6	Methods - Study population and Clinical assessment [Page 5] <u>Participants of the study fulfilled one of the following eligibility criteria:</u> i) relatives of a patient with definite FD diagnosis. ii) patient cases with nephrological, cardiac or neurological symptoms suspicious of FD diagnosis from various medical units all over Greece. - Additional genotyping of 145 apparently healthy subjects. - The source of participants selection was their medical records (relation with a FD patient, patients with FD related symptoms), analysed in collaboration with their treating physicians. - Patients follow-up in collaboration with their treating physicians. Revaluation of the clinical status of each selected individual.
Variables	7	Clinical assessment - Laboratory evaluation - Genotyping – [Pages 5-6]

Variables used in the study:

- i) Heart or kidney disease, cerebrovascular events, death at young age and respective causes of death.
- ii) FD related cardiac, renal and neurological signs and symptoms.
- iii) Familiar relationship to a FD patient.
- iv) Enzyme α -Gal A activity in all male subjects.
- v) Lyso-Gb₃ in all subjects.
- vi) Plasma and urine Gb₃ concentration in selected cases.
- vii) Presence of a *GLA* mutation.
- viii) Verification of novel mutations.
- ix) Evaluation of kidney biopsies.

Diagnostic criteria [Page 6]

The diagnosis of FD was posed according to the recently published criteria of the disease [Biegstraaten et al. 2015]

Data sources/ measurement	8	Clinical assessment - Laboratory evaluation - Genotyping – page 5-6 <u>Method of assessment for each variable:</u> <ul style="list-style-type: none"> i) Medical history. ii) Physical examination. iii) Pedigree analysis. iv) In DBS by tandem mass spectrometry. v) In DBS by HPLC and tandem spectrometry. vi) Tandem mass spectrometry. vii) Genotyping. viii) Standard Sanger sequencing using Variant Reporter software v1.1 (Applied Biosystems). ix) Optical and electron microscopy.
Bias	9	Study population [Page 5] Cross examination for the presence of D313Y mutation by genotyping of 145 apparently healthy subjects. Laboratory evaluation [Page 5] Measurement of α -Gal A activity only in male subjects. Genotyping [Page 6] Exclusion of common polymorphisms (UCSC Common SNPs) by bioinformatic analysis using PhyloP, SIFT, Grantham and PolyPhen tools, in comparison to their global (1000 Genomes Global Minor Allele Frequency, ExAC) and European frequency (5000 Exomes European Minor Allele Frequency).
Study size	10	Study population [Page 5] The cohort size of 62 subjects was arrived after selection of interesting patient cases between families of 9 unrelated patients with definite diagnosis of FD as well as amongst cases with nephrological, cardiac or neurological symptoms suspicious of this diagnosis. Eighteen family members of the last cases were also examined after the confirmation of diagnosis.
Quantitative variables	11	Laboratory evaluation [Page 5]

In DBS, α -Gal A activity was measured in all male subjects by tandem mass spectrometry, lyso-Gb₃ in all subjects by HPLC and tandem spectrometry as well as plasma and urine Gb₃ concentration in selected cases by tandem mass spectrometry.

Statistical methods	12	Age of each patient or patient group is presented either as an absolute number or as mean value \pm the Standard Deviation (mean \pm SD).
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Results

Participants	13	Results [Page 6] 62 subjects were potentially eligible for participation in the study and were genotyped for a <i>GLA</i> mutation, clinically analysed and under follow-up for a significant period of time. Abstract [page 3] The 62 subjects were selected between 3 categories of patient cases: i) Family members of unrelated patients with definite FD diagnosis. ii) Clinically suspected cases. iii) Family members of the previous category.
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Descriptive data	14	Abstract [Page 3] i) 25 family members of 9 unrelated patients with definite FD diagnosis. ii) Ten clinically suspected cases (nephrological, cardiac or neurological symptoms suspicious of FD diagnosis) from various medical units all over Greece. iii) 18 family members of the previous category. Results [Pages 6-11] i) Patients carrying the D313Y mutation: 17 (54 \pm 14, range 27– 78 years). [page 6] ii) Male patients carrying the D313Y mutation: 7 (61 \pm 11, range 45– 78 years). [page 6] iii) Female patients carrying the D313Y mutation: 10 (49 \pm 15, range 27– 70 years). [page 6] iv) Patients carrying novel <i>GLA</i> mutations: 16 members (42 \pm 19, range 1– 73 years) of 5 unrelated families, all fulfilling the diagnostic criteria of a definite diagnosis of FD. [page 8] v) Patients identified with the novel c.835C>T mutation: 4 members (30 \pm 24, range 1– 60 years) of a Greek family. Proband of the family: a 31-year-old male with typical FD clinical and laboratory phenotype. [page 8,9] vi) Patients identified with the novel c.280T>A mutation: 4 members (46 \pm 11, range 30– 56 years) belonging to another Greek family. Proband of the family: a 48-year-old female with typical FD clinical and laboratory phenotype. [page 9] vii) Patients identified with the novel c.924A>C mutation: 3 members (44 \pm 26, range 17– 69 years) of a Greek family. Proband of the family: a 46-year-old male with typical FD clinical and laboratory phenotype. [page 9,10] viii) Patients identified with the novel c.511G>A mutation: 2 members (37 \pm 4, range 34– 39 years) of an Albanian family living in Greece. Proband of the family: a 39-year-old male with typical FD clinical and laboratory phenotype. [page 10] ix) Patients identified with the novel c.453C>G mutation: 3 members (55 \pm 16, range 42– 73 years) of an other Greek family. Proband of the family: a 50-year-old male with typical FD clinical and laboratory phenotype. [page 10,11]
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Outcome data	15	Results [pages 6-11] i) Six (all with definite FD) out of the 62 genotyped subjects were carrying four previously described <i>GLA</i> mutations. [page 6]
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- ii) The D313Y mutation was revealed in 17 individuals, 7 males and 10 females. [page 6]
 iii) The novel c.835C>T, c.280T>A, c.924A>C, c.511G>A and c.453C>G *GLA* mutations were detected in 16 members of 5 unrelated families.

Main results	16	<p>Results [pages 6-11]</p> <p>i) Six (all with definite FD) out of the 62 genotyped subjects were carrying four previously described <i>GLA</i> mutations. [page 6]</p> <p>ii) The D313Y mutation was revealed in 17 individuals, 7 males and 10 females. [page 6]</p> <p>iii) The D313Y mutation was revealed in none of the healthy subjects. [page 6]</p> <p>iv) The diagnosis of FD was definitely posed in 5 carriers of the D313Y mutation. [page 6-8]</p> <p>v) Two D313Y mutation carriers were patients presenting no FD related signs or symptoms. [page 8]</p> <p>vi) Eight D313Y mutation carriers were patients presenting other FD signs mainly neurological that, however, can not document a definite diagnosis of FD. [page 8]</p> <p>vii) Two D313Y mutation carriers were apparently healthy. [page 8]</p> <p>viii) The diagnosis of FD was definitely posed in all carriers of the novel mutations reported. [page 8-11]</p>
Other analyses	17	<p>i) Bioinformatics analysis of every <i>GLA</i> mutation reported, by (PolyPhen-2) or (SIFT) systems. [page 12-13]</p> <p>ii) Novel mutations verification by standard Sanger sequencing using Variant Reporter software v1.1 (Applied Biosystems). [page 6]</p>
Discussion		
Key results	18	<p>i) Five novel <i>GLA</i> mutations causing classical Fabry Disease are reported. [page 14]</p> <p>ii) Strong evidence that the D313Y mutation could be pathogenic is offered. [page 14]</p>
Limitations	19	The main limitation is the lack of detailed clinical data in older participants. [page 4]
Interpretation	20	<p>Discussion [Pages 11-13]</p> <p>- Five of seventeen carriers of the D313Y mutation proved as suffering definite FD according to the recently published criteria of the disease. The presentation of the disease in our patients indicates that the mutation results in a milder phenotype, with later onset of symptoms. [page 12]</p> <p>- Contradicting results about the pathogenicity of this mutation have been reported in the literature since its first description on 1993. [page 11-12]</p> <p>- The mutation has been detected in many series of patients presenting signs of FD. However, Niemann et al. describes this variant as non pathogenic, although his two patients were presenting decreased α-gal A activity. Similarly, Oder et al. supports that the D313Y genotype does not lead to severe organ manifestations as seen in genotypes known to be causal for classical FD and Froissart et al. [i] characterizes the mutation as “pseudodeficient allele” implying that it is a sequence variant which encodes an enzyme that is transported to the lysosomes, where it has about 75% of normal enzymatic activity. The D313Y mutation has been also referred as polymorphism. [page 11-12]</p> <p>- The vast majority of the D313Y patients of the study were presenting with neurological symptoms and signs. [page 12]</p> <p>- Kidney biopsy is significant for a FD diagnosis in patients with renal manifestations of the disease [page 7, 9-11]</p>

- The pathogenicity of the five novel mutations reported has been undoubtedly proved as all carriers suffer definite FD. [page 13]

Generalisability	21	Discussion [Pages 11-13]
		Conclusion [Page 13-14]

The study underlines the significance of :

- i) Diagnosis of FD and possible treatment of patients carrying the 5 novel and the D313Y *GLA* mutations.
- ii) Family members genotyping.
- iii) Newborn screening and genetic counselling.
- iv) Avoiding misdiagnoses and crucial delays of diagnosis and treatment of the disease.

The study confirmed the facts that:

- i) Heterozygous females may develop mild to severe FD.
- ii) Misdiagnosis is common in FD.
- iii) Genotype-phenotype correlation does not exist even among the members of the same families.

Other information

Funding	22	Funding [Page 19]
		The study has been partially supported by a grant from the Research Committee of the University of Thessaly.

BMJ Open

Fabry disease due to D313Y and novel GLA mutations

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Keywords:	Fabry disease, D313Y GLA mutation, Novel GLA mutations, Kidney biopsy, Misdiagnosis

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Manuscripts

1 Fabry disease due to D313Y and novel GLA mutations

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27 **Abstract**

28 **Objectives:** Our aim is to report four novel *GLA* mutations resulting in FD and provide
29 evidence of pathogenicity of the D313Y mutation regarding which contradictory data have
30 been presented in the literature.

31 **Setting and participants:** 25 family members of nine unrelated patients with definite FD
32 diagnosis, ten clinically suspected cases and eighteen members of their families were included
33 in this polycentric cohort study.

34 **Primary and secondary outcome measures:** Genotyping and measurement of lyso-Gb₃ was
35 performed in all individuals. The α -Gal A activity was measured in all males as well as
36 plasma and urine Gb₃ concentration in selected cases. Optical and electron microscopy was
37 performed in kidney biopsies of selected patients. All the above were evaluated in parallel
38 with the clinical data of the patients.

39 **Results:** Fourteen new cases of FD were recognised, four of which were carrying already
40 described *GLA* mutations. Four novel *GLA* mutations, namely c.835C>T, c.280T>A,
41 c.924A>C, and c.511G>A, resulting in a classical FD phenotype were identified. Moreover,
42 FD was definitely diagnosed in five patients carrying the D313Y mutation. Eight D313Y
43 carriers were presenting signs of FD despite not fulfilling the criteria of the disease, two had
44 no FD signs and two others were apparently healthy.

45 **Conclusions:** Four novel *GLA* pathogenic mutations are reported and evidence of
46 pathogenicity of the D313Y mutation is provided. It seems that the D313Y mutation is
47 related with a later-onset milder than the typical phenotype with normal lysoGb₃
48 concentration. Our study underlines the significance of family members genotyping and
49 newborn screening in avoiding misdiagnoses and crucial delays of diagnosis and treatment of
50 the disease.

Strengths and limitations of this study

- This is the largest series in the literature of clinically evaluated male and female carriers of the *GLA* D313Y mutation supporting its possible pathogenicity that occasionally has been proved by renal biopsy.
- Novel *GLA* mutations resulting to a classical Fabry disease phenotype are presented.
- The main limitation is the lack of detailed clinical data in older participants.
- Biopsies of affected organs, the gold standard of definite diagnosis, are not available in all cases.

INTRODUCTION

FD or Anderson-Fabry disease is an X-linked inherited metabolic disorder, that results from mutations in the α -gal A gene (*GLA* gene), leading to reduction of the enzyme activity and subsequent accumulation of Gb₃ (or GL-3) in plasma, urine and cellular lysosomes throughout the body. These depositions cause a multisystemic pathology with life-threatening manifestations, including renal failure, cardiac and cerebrovascular disease [1, 2].

More than 900 currently known *GLA* mutations have been identified [3, 4], as causing a variety of clinical manifestations. Most of them are unique to a family (private) and therefore genotype-phenotype correlation is limited [5]. Diagnosing FD is challenging due to the range of disorders that mimic the disease and the great variety of atypical clinical presentations. As a result underdiagnosis and misdiagnosis of FD lead to late diagnosis, delays in appropriate treatment and a subsequent negative prognosis [6]. Human genetic analysis must be performed, in order to exclude or verify a mutation of the *GLA* [7]. Once a diagnosis has been made, biochemical and molecular genetic analysis, as well as genetic counselling, should be made available to all family members [8]. A detailed pedigree analysis for each patient

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74 presenting with FD is crucial [9], as it can inform the diagnosis of the proband and the
75 identification of all at-risk relatives [10].

76 Our aim is to report four novel *GLA* mutations resulting in FD and provide evidence of
77 pathogenicity of the D313Y mutation regarding which contradictory data have been presented
78 in the literature.

79 **METHODS**

80 **Study design and setting**

81 This is a polycentric population screening study of individuals from Greece either
82 demonstrating phenotypic traits suggestive for FD or belonging to families of patients with
83 definite FD diagnosis and fulfilling anyone of the following inclusion criteria:

- 84 • Definite diagnosis of FD.
- 85 • Nephrological, cardiac or neurological symptoms suspicious of FD.
- 86 • Relatives of patients with definite FD diagnosis.

87 A cohort of 62 subjects from 19 unrelated families was involved in the study. Twenty-
88 five of them were relatives of 9 patients with definite FD diagnosis and 18 were relatives of
89 10 individuals recruited as carriers of FD traits in whom a *GLA* mutation was detected. The
90 presence of the D313Y mutation in Greek population was examined by genotyping of 145
91 apparently healthy subjects (70 males, 75 females). Written informed consent was obtained
92 from each subject or an accompanying relative, where legally appropriate. The study was
93 approved by the institutional review board of the University of Thessaly, Larissa.

94 **Clinical assessment**

95 Patients' medical records were revaluated and a detailed medical history of the family
96 members was obtained especially in regard with heart or kidney disease, cerebrovascular
97 events, death at young age and respective causes of death. All study participants underwent

physical examination particularly focused on cardiac, renal and neurological signs and symptoms. A detailed pedigree was constructed for every family and newborn screening was performed once.

Laboratory evaluation

In DBS, we measured α -Gal A activity in all male subjects by tandem mass spectrometry [11], lyso-Gb₃ in all subjects by HPLC and tandem spectrometry [12] as well as plasma and urine Gb₃ concentration in selected cases by tandem mass spectrometry [13].

Optical and electron microscopy was performed for the study of kidney biopsies occasionally.

Genotyping

Genomic DNA was extracted from peripheral blood using the iPrep Pure Link DNA blood kit (Invitrogen, ThermoFisher, USA) according to manufacturer's instructions. All coding regions and exon-intron splice junctions of the *GLA* gene were analysed in a targeted custom next-generation sequencing (NGS) platform (Ampliseq custom panel, Thermo Scientific). Analysis of primary data was conducted with Ion Reporter software v.5.2 (Thermo Scientific). Common polymorphisms (UCSC Common SNPs) were excluded and pathogenicity of variations was predicted by bioinformatic analysis using PhyloP, SIFT, Grantham and PolyPhen tools, in comparison to their global (1000 Genomes Global Minor Allele Frequency, ExAC) and European frequency (5000 Exomes European Minor Allele Frequency). The characterization of variants was based on the recommendations of the American College of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology [14].

Novel variants were verified by PCR in combination with Sanger sequencing. Amplification of *GLA* exons (including exon-intron boundaries) was performed in 5 reactions

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3 122 corresponding to exons 1, 2, 3, 4, and 5–7, using the primers included in Supplementary
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5 123 Material. A total of 100-200 ng of genomic DNA was amplified by PCR in a 30 μ L reaction
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7 124 mixture using 200 μ M of each deoxynucleoside triphosphate, 30 pmol of each primer, 1.5
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9 125 mM MgCl₂ and 1.0 U Taq polymerase (Invitrogen, Thermofisher, USA) in a 10x buffer
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11 126 supplied by the manufacturer. Reaction conditions were as following: For exons 1-3: 94 °C
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13 127 for 2 min, followed by 30 cycles of 30 sec at 94 °C, 30 s at 58 °C, 30 s at 72 °C, and a final
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15 128 extension at 72 °C for 5 min. For exons 4 and 5–7: 94 °C for 2 min, followed by 32 cycles of
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17 129 30 sec at 94 °C, 30 sec at 54 °C, 30 sec for exon 4 or 75 sec for exons 5-7 at 72 °C, and a final
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19 130 extension at 72 °C for 5 min. All PCR reactions were carried out in the Veriti 96-Well
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21 131 Thermal Cycler (Applied Biosystems, Thermofisher, USA) PCR engine apparatus and the
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23 132 emerging PCR products were purified using the PureLink PCR Purification Kit system
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25 133 (ThermoFisher Scientific, USA). Sequencing was performed using the primers described in
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27 134 Table X, using a 3730 DNA Analyzer (Applied Biosystems, Thermofisher, USA) and BigDye
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29 135 Terminator DNA sequencing kit (Applied Biosystems, Thermofisher, USA) according to
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31 136 manufacturer's instructions.
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37 137 **RESULTS**
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39 138 Nine (all with definite FD) out of the 62 genotyped subjects were carrying five
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41 139 previously described *GLA* mutations: c.334C>T (p.Arg112Cys, R112C), c.644A>G
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43 140 (p.Asn215Ser, N215S), c.1153A>C (p.Thr385Pro, T385P), c.453C>G (p.Tyr151Ter,
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45 141 Y151X) and c.782G>T (p.Gly261Val, G261V).
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49 142 The c.937G>T (p.Asp313Tyr, D313Y, NM_000169.2) mutation was revealed in
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51 143 seventeen individuals (54 \pm 14, range 27– 78 years), seven males (61 \pm 11, range 45– 78 years)
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53 144 and ten females (49 \pm 15, range 27– 70 years) but in none of the healthy subjects. Patients'
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55 145 clinical and laboratory findings are presented in Table 1. All male patients (61 \pm 11, range 45–
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57 146 78 years) presented with an α -gal A activity decreased in a range of 56.2-87.5% comparing to
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normal with the exception of one of them in whom α -gal A activity was within normal range. LysoGb₃ concentration was found normal (range 0.8-1.7 ng/mL) in all patients, while plasma and urine Gb₃ concentration was varying as shown in Table 1.

The diagnosis of FD was definitely posed according to the recently published criteria of the disease [15], in five carriers of this mutation (54±9, range 45– 65 years). The first of them (patient no.1, Table 1), a 52-year-old man was initially diagnosed at the age of 48 with CKD stage III and was not presenting cardiac or other FD signs. Kidney biopsy performed because of non-nephrotic proteinuria, microscopic haematuria and raised serum creatinine, revealed focal and segmental glomerulosclerosis (FSGS) – collapsing variant. At that time enzyme activity and plasma lyso-Gb₃ concentration were normal. Three years after initial presentation the patient was suffering end stage renal disease and extreme acroparesthesias. Revaluation of kidney biopsy by higher magnification uncovered focal cytoplasmic microvacuolization of enlarged podocytes (Fig.1) while a decreased by 50% α -gal A activity and an increased plasma (7.52 nmol/mL, reference: 0.8-4.52) and urine Gb₃ concentration (147.49 nmol/g, reference: <29.00) were detected. The patient commenced dialysis and ERT with rapid clinical improvement.

A second carrier of the D313Y mutation was a 46-year-old female (patient no. 4, Table 1) who had suffered a TIA and two ischaemic strokes that had been considered of unknown origin. On revaluation, one year after the last stroke the patient appeared with microalbuminuria, oedema and acroparesthesias on both hands. The microalbuminuria was duplicated after three months and acroparesthesias worsened. Gb₃ concentration was pathological in urine (54.08 nmol/g) but normal in plasma. LysoGb₃ concentration was normal at that time and remained stable for one more year.

The mother of the above patient (patient no. 5, Table 1), 65 years old, had been diagnosed at the age of 50 with MS and was receiving relative medication, without clinical

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benefit. She was also receiving treatment for pain in extremities that were attributed to RA. During the recent years the patient suffered mobility impairment (reported as spastic quadriplegia after a neurological examination), depression and dementia. The evaluation of the patient revealed pathological plasma Gb₃ concentration (4.7 nmol/mL), WML on brain MRI and normal kidney function. LysoGb₃ concentration was normal and remained so during a 6-month-follow up.

Another carrier of the D313Y mutation was a 45-year-old male (patient no.7, Table 1) on dialysis due to CKD by the age of 25. No kidney biopsy was performed at that time. He reports episodes of haematuria during childhood and adolescence, attributed at that time to vesicoureteral reflux. Enzyme activity was slightly decreased (2.4 µmol/l/h, reference: ≥2.6) and plasma lyso-Gb₃ concentration was normal. Brain MRI revealed WML and vertebrobasilar vessel changes. Moreover, increased echogenicity of cardiac interventricular septum on the echocardiogram and sensorineural hearing loss of higher frequencies. The patient commenced ERT.

The last patient with D313Y mutation definitely diagnosed as suffering FD was a 60-year-old female (patient no.17, Table 1) who was presenting cornea verticillata corneopathy, WML and ischaemic infarcts on brain MRI despite that no stroke is reported, acroparesthesias and GI symptoms (pain-diarrhoea) since adolescence, hypohidrosis, hearing loss and LVH.

Among the remaining twelve D313Y mutation carriers there were two patients with no FD signs and eight patients (55±17, range 27–78 years) presenting other FD signs mainly neurological that, however, cannot document a definite diagnosis of FD. Especially patient no.6, Table 1 (62-year-old female) suffers acroparesthesias and GI symptoms since adolescence and presents with lysoGb₃ at 1.7 ng/ml (reference: ≥ 1.8). Accordingly patient no. 16, Table 1 (64-year-old male) presents with CKD, diabetes mellitus and hearing loss,

196 while on brain MRI presents multiple ischaemic infarcts despite that no stroke is reported.
197 The remaining two D313Y mutation carriers are apparently healthy.

198 Novel *GLA* mutations (4) (Fig. 2) were detected in thirteen members (39±18, range 1–
199 69 years) of four unrelated families (Table 2), all fulfilling the diagnostic criteria of a definite
200 diagnosis of FD [15]. The c.835C>T mutation (p.Gln279Ter, Q279X) in exon 6 of the *GLA*
201 gene was identified in four members (30±24, range 1–60 years) of a Greek family. The
202 proband, a 31-year-old male, was presented at the age of 23 with proteinuria (3.5 gr/24 h),
203 microscopic haematuria, slightly deteriorated kidney function, right bundle brunch block,
204 mild hypertension and angiokeratomas in the arms and the loin area. He reported pain in the
205 extremities especially during infections and inability to sweat. The α -gal A activity was close
206 to zero. Cardiac MRI showed moderate LVH. The kidney biopsy showed cytoplasmic
207 vacuolization and extended lysosomal accumulations in all types of kidney cells,
208 especially in the podocytes (Fig. 3). The patient is currently under ERT with beneficial
209 results in regard with kidney function, proteinuria and pain. Three other members of the
210 family were revealed having with the same mutation and all presented clinical signs of
211 FD. The proband's mother (60 years old) reported a possible TIA at the age of 32. Kidney
212 examination showed albuminuria (> 500 mg/24 h), microscopic haematuria and normal
213 kidney function. Cardiac MRI revealed severe LVH (cardiac interventricular septum over
214 19mm) and on skin examination she showed angiokeratomas in the arms. Her mother (the
215 proband's grandmother) had died at the age of 62, due to cardiac arrest. She suffered from
216 severe LVH and acroparesthesias, which at that time were attributed to Raynaud's
217 phenomenon. The proband's sister (27-year-old) has elevated lyso-Gb₃ concentration (2.7
218 ng/ml) and screening of her newborn daughter revealed the mutation too.

219 Four patients (46±11, range 30– 56 years) belonging to another Greek family were
220 carrying the c.280T>A (p.Cys94Ser, C94S) mutation. The proband, a 48-year-old female, was

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221 diagnosed with FD a year before after presenting increased lyso-Gb₃ concentration (5.4
222 ng/ml), increased plasma Gb₃ concentration (6.14 nmol/ml), microalbuminuria of no other
223 origin, cornea verticillata corneopathy, acroparesthesias in both hands and dyshidrosis.
224 Clinical and laboratory data of the other suffering members of the family are presented in
225 Table 2.

226 The c.924A>C mutation (K308N - p.Lys308Asn) in exon 6 of the *GLA* gene was
227 identified in three members (44±26, range 17– 69 years) of a Greek family. The proband, a
228 46-year-old male, was presented at the age of 34 with albuminuria (0,5-0,6 gr/24h),
229 microscopic haematuria since ten years and slight hypertension. The kidney function was
230 normal. Kidney biopsy showed slight mesangial proliferative damages and cytoplasmic
231 microvacuolization of podocytes. The α-gal A activity was almost zero. After 10-year-
232 follow up he suffered of LVH and proteinuria (1.8 gr/24h). The patient is currently under
233 ERT. His mother and daughter carry the same mutation. The probands grandmother had
234 died at the age of 68, suffering of severe LVH and end-stage heart failure.

235 Lastly, the c.511G>A mutation (G171S - p.Gly171Ser) in exon 3 of the *GLA* gene was
236 identified in two members (37 ± 4, range 34– 39 years) of an Albanian family living in
237 Greece. The proband, a 39-year-old male, was diagnosed at the age of 32 with severely
238 deteriorated kidney function and proteinuria. No biopsy was performed at that time, due to the
239 small size of the kidneys. After nearly a year he presented with severe clinical and laboratory
240 findings of acute renal failure and need of dialysis. Normal kidney function was never
241 restored. FD was definitely diagnosed at the age of 37, as the α-gal A activity was extremely
242 low and lyso-Gb₃ concentration was 11.9 ng/ml. The patient is suffering of LVH, increased
243 pulmonary artery diameter, dilatation of the ascending aorta and aortic valve stenosis, because
244 of which he underwent a valve replacement surgery. Ophthalmological evaluation indicated
245 lipid deposition with blurriness of the cornea. The patient is currently under ERT. His 34-

246 year-old brother was identified with the same mutation and has extremely low α -gal A
247 activity, lyso-Gb3 concentration 12.9 ng/ml and albuminuria of no other aetiology.

248 **DISCUSSION**

249 **The D313Y mutation**

250 Contradicting results about the pathogenicity of this mutation have been reported in the
251 literature since its first description on 1993 [16]. The mutation has been detected in many
252 series of patients presenting signs of FD [17, 18, 19, 20, 21, 22, 23, 24]. However, Niemann
253 et al. [25] describes this variant as non pathogenic, although his two patients were presenting
254 decreased α -gal A activity. Similarly, Oder et al. [26] supports that the D313Y genotype does
255 not lead to severe organ manifestations as seen in genotypes known to be causal for classical
256 FD and Froissart et al. [27] characterizes the mutation as “pseudodeficient allele” implying
257 that it is a sequence variant which encodes an enzyme that is transported to the lysosomes,
258 where it has about 75% of normal enzymatic activity. The D313Y mutation has been also
259 referred as polymorphism [19], despite that, according to the ExAC and 1000 Genomes
260 databases, its frequency in the World and European population is below 1%. Finally, it must
261 be mentioned that by bioinformatics analysis this mutation is predicted as probably damaging
262 (PolyPhen-2) or damaging (SIFT).

263 In our study five of seventeen carriers of the D313Y mutation (54 ± 9 , range 45– 65
264 years) proved as suffering definite FD according to the recently published criteria of the
265 disease [15]. The presentation of the disease in our patients indicates that the mutation results
266 in a milder phenotype, with later onset of symptoms. This phenotype, also including milder
267 mono- or oligosymptomatic cases [19], is characterised as atypical or type 2 [28]. The late
268 onset of clinical symptoms and the milder than the typical phenotype of FD in these patients
269 can be partly explained by the high α -gal A residual activity, since there is evidence that the

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mutated α -gal A reaches intracellularly the lysosomes [29]. Actually, in all but one male D313Y carriers of our study α -gal A activity was decreased. Another reason for the decreased activity of the D313Y enzyme in plasma could be a functional intolerance to blood plasma neutral pH conditions. This effect is irreversible and, once in contact with a neutral or basic pH environment D313Y enzyme remains inactive, even if transferred to optimal pH [21, 29].

Interestingly, the vast majority of our D313Y patients were presenting with neurological symptoms and signs. Moreover, one of them was misdiagnosed as MS while two carriers of the mutation, in whom definite FD diagnosis has not been established as yet, are diagnosed and treated as MS. Similarly the detailed study of the renal biopsy in one of our cases underlies the significance of early detection of Fabry specific findings in cases with FSGS.

Novel mutations

The pathogenicity of the above mentioned novel mutations has been undoubtedly proved as all carriers suffer definite FD. Of them the c.835C>T (p.Gln279Ter, Q279X) is a nonsense mutation causing an interruption of the reading frame by a premature stop codon, which results in a truncated protein. A truncated protein and the subsequent loss of its functionality is strong evidence that the mutation is probably pathogenic for FD [14]. No amino acid change at this position has ever been described in NCBI and Fabry Database (<http://fabry-database.org/>) [30].

As far as the c.280T>A (C94S), c.924A>C (K308N), c.511G>A (G171S) mutations are considered, bioinformatics analysis supports their disease causing effect as they are predicted as probable damaging (PolyPhen-2) and/or damaging (SIFT). A further evidence of their pathogenicity could be the fact that the same aminoacid changes at the corresponding positions but in different nucleotides have already been described as disease causing [31, 32, 33].

CONCLUSIONS

We report four novel *GLA* mutations causing FD. Moreover, we offer strong evidence that the D313Y mutation could be pathogenic. It seems that this mutation is related with a later-onset milder than the typical phenotype with normal lysoGb₃ concentration.

Additionally our study underlines the significance of family members genotyping, newborn screening and genetic counselling in avoiding misdiagnoses and crucial delays of diagnosis and treatment of the disease. Finally we confirmed the fact that heterozygous females may develop mild to severe FD as well as that genotype-phenotype correlation does not exist even among the members of the same families.

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Table 1. Characteristics of patients carrying the D313Y mutation. Plasma and urine Gb₃ concentration was measured only in patients 1, 4 and 5.

Patient / Gender / Age (years)	FD related clinical findings	Previous diagnosis
1 / M / 52	End stage renal disease – dialysis, acroparesthesias, renal cysts, elevated plasma and urine Gb ₃ concentration	FSGS nephropathy
2 / F / 30	Healthy	no
3 / F / 70	Healthy	no
4 / F / 46	Three strokes of unknown origin, WML on brain MRI, micro-albuminuria, oedema, acroparesthesias, elevated urine Gb ₃ concentration	no
5 / F / 65	Multiple WML on brain MRI, depression, elevated plasma Gb ₃ concentration	MS, RA, spastic quadriplegia
6 / F / 62	Acroparesthesias, GI symptoms since adolescence	no
7 / M / 45	End stage renal disease on dialysis, WML and vertebrobasilar vessel changes on brain MRI, increased cardiac interventricular septum echogenicity, hearing loss of higher frequencies	Nephropathy of unknown origin
8 / M / 62	End stage renal disease on dialysis	no
9 / F / 36	WML on brain MRI, acroparesthesias	MS
10 / M / 78	End stage renal disease on dialysis	RA
11 / F / 47	LVH, CKD	no
12 / M / 68	No FD signs	Myopathy
13 / F / 46	No FD signs	NMO
14 / F / 27	WML on brain MRI	MS
15 / M / 61	End stage renal disease on dialysis	Diabetic nephropathy
16 / M / 63	CKD, hearing loss, multiple ischaemic infarcts on brain MRI	no
17 / F / 59	Cornea verticillata, hearing loss, LVH, acroparesthesias, GI symptoms (pain – diarrhoea) since adolescence, hypohidrosis, T2 -WML or ischaemic infarcts on brain MRI	no

Table 2. Clinical characteristics of patients with the five novel mutations.

Mutation (NM_000169.2)	Clinical data
c.835C>T p.Gln279Ter Q279X	CKD, haematuria, proteinuria, pain in extremities, dyshidrosis, angiokeratomas, zero activity of a-gal A, LVH, kidney biopsy consistent with FD, TIA, pathological elevated lyso-Gb ₃
c.280T>A p.Cys94Ser C94S	Microalbuminuria, end stage renal disease – dialysis, cornea verticillata corneopathy, acroparesthesias, dyshidrosis, LVH, WML on brain MRI, zero activity of a-gal A, pathological elevated lyso-Gb ₃ , pathological elevated Gb ₃ in plasma/urine
c.924A>C p.Lys308Asn K308N	CKD, haematuria, kidney biopsy findings related to FD, zero activity of a-gal A, LVH
c.511G>A p.Gly171Ser G171S	Proteinuria, end stage renal disease – dialysis, LVH, valvulopathy, cornea verticillata corneopathy, extremely low activity of a-gal A, pathological elevated lyso-Gb ₃

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Figure 1. Optical microscopy findings of renal biopsy from a male FD patient carrying the D313Y mutation of the *GLA*. (a) Glomerulus with segmental sclerosis - PAS x400. (b) Segmental sclerosis with features of the “collapsing” variant (arrow) - Jones’ silver x400. (c) Pale appearing glomerulus with a small area of sclerosis adhering to Bowman’ s capsule (arrow) - PAS x400. (d) Cytoplasmic microvacuolization of podocytes (arrow), suggestive of FD - PAS x400.

Figure 2. Sanger confirmation of novel mutations.

Figure 3. Electron microscopy findings of renal biopsy from a male FD patient carrying the Q279X mutation. Multi-lamellated myelin figures (“zebra” bodies), typical finding of FD, are marked with black arrows in (a) methylene blue semithin section, (b) tubular cells and a fibroblast and (c,d) podocytes.

Abbreviations

α -gal A: Enzyme α -galactosidase A; ACMG: American College of Medical Genetics and Genomics; CKD: Chronic kidney disease; DBS: Dried blood spot; ERT: Enzyme replacement therapy; FD: Fabry disease; FSGS: focal and segmental glomerulosclerosis; GI: Gastrointestinal; GLA: α -galactosidase A gene; GL-3 or Gb3: globotriaosylceramide; LVH: Left ventricular hypertrophy; lyso-Gb3: globotriaosylsphingosine; MRI: Magnetic resonance imaging; MV: mean value; MS: Multiple sclerosis; NMO: Neuromyelitis optica; RA: Rheumatoid arthritis; SD: Standard deviation; TIA: Transient ischemic attack; UT: Under treatment; WML: White matter lesions.

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Availability of data and materials

All data supporting our findings are included in the manuscript.

Authors' contributions

KK: collection, analysis and interpretation of the data, pedigree analysis, literature review, drafting and revision of the manuscript. KS: Renal biopsy and analysis on electron microscope. PP: Renal biopsy and analysis on optical microscope. MS, MZ and GL: genotyping and bioinformatics analysis. KS, PP, EM, PK, CK, AO, JK: treating physicians of patients. AEG: design and coordination of the study, revision of the manuscript. All authors approved the final version of the manuscript.

Competing interests

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349 research grants from Shire. The authors MZ, GL, MS, EM, PK, JK, report no competing
350 interests.

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353 University of Thessaly.

354 **Consent for publication**

355 Written informed consents for this publication were obtained from patients. A copy of the
356 consent form is available for review by the Editor of this journal.

357 **Ethics approval and consent to participate**

358 This report was approved by the institutional review board of the University of Thessaly,
359 Larissa. Written informed consents for participation were obtained from patients.

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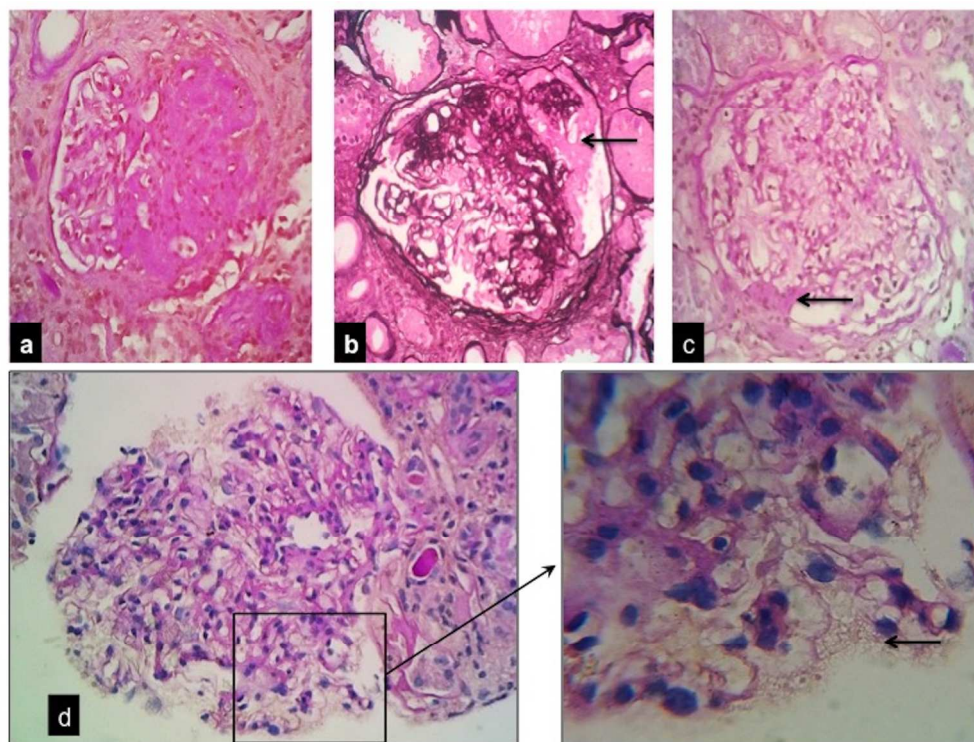


Figure 1. Optical microscopy findings of renal biopsy from a male FD patient carrying the D313Y mutation of the GLA. (a) Glomerulus with segmental sclerosis - PAS x400. (b) Segmental sclerosis with features of the "collapsing" variant (arrow) - Jones' silver x400. (c) Pale appearing glomerulus with a small area of sclerosis adhering to Bowman's capsule (arrow) - PAS x400. (d) Cytoplasmic microvacuolization of podocytes (arrow), suggestive of FD - PAS x400.

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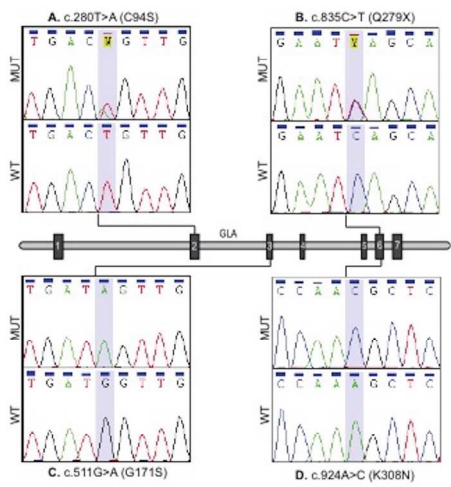


Figure 2. Sanger confirmation of novel mutations.

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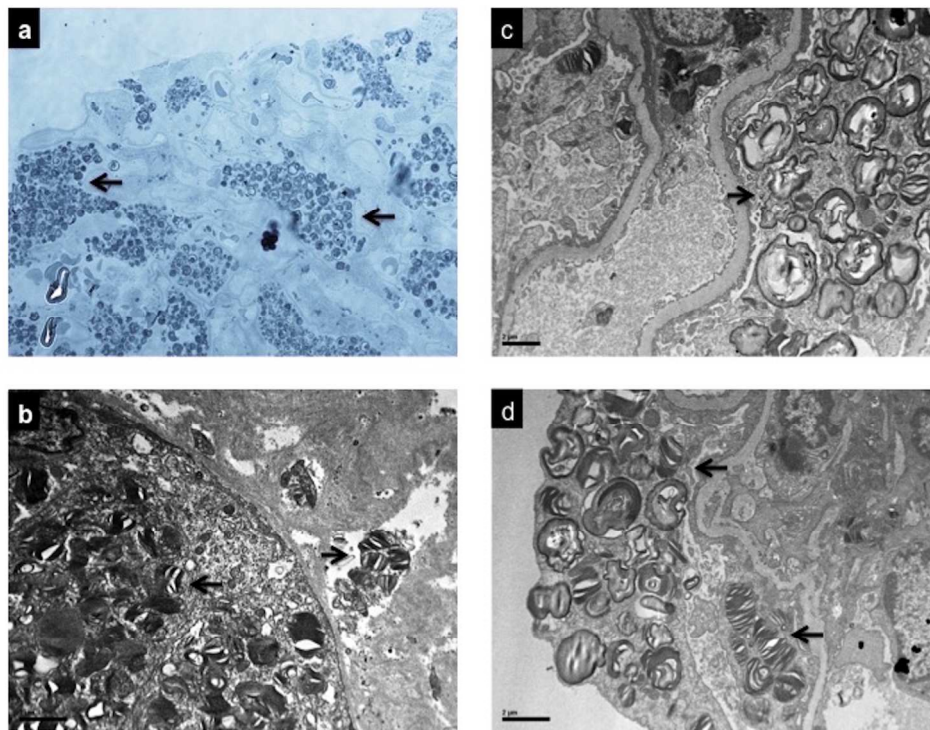


Figure 3. Electron microscopy findings of renal biopsy from a male FD patient carrying the Q279X mutation. Multi-lamellated myelin figures ("zebra" bodies), typical finding of FD, are marked with black arrows in (a) methylene blue semithin section, (b) tubular cells and a fibroblast and (c,d) podocytes.

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Supplementary file: Primer sequences for amplification and sequencing of the *GLA* gene.

Fragment	Sequence	Size (bp)
Genomic DNA amplification		
Exon 1	Forward: 5'- CCCAGTTGCCAGAGAAACAATAAC-3' Reverse: 5'-AGACTCTCCAGTTCCCCAAACAC -3'	391
Exon 2	Forward: 5'-CCCAAGGTGCCTAATAAATGG -3' Reverse: 5'-CCATGAGGGCTGTTTCTAA -3'	337
Exon 3	Forward: 5'-CGCAGCCTGGAATGGTTCTCTC -3' Reverse: 5'-CTCAGCTACCATGGCCTCA -3'	323
Exon 4	Forward: 5'-AGCTGGAAATTCATTTCTTT -3' Reverse: 5'-TTGGTTTCCTTTGTTGTCA -3'	212
Exons 5-7	Forward: 5'-AAACTCAAGAGAAGGCTACAAGT -3' Reverse: 5'-AAAAAGGTGGACAGGAAGTAGTAGT 3'	1280
Sequencing Primers		
Exon 1 forward	5'- CCCAGTTGCCAGAGAGAAACAATAAC-3'	
Exon 2 forward	5'-CCCAAGGTGCCTAATAAATGG -3'	
Exon 3 forward	5'-CGCAGCCTGGAATGGTTCTCTC -3'	
Exon 4 forward	5'-TTGGTTTCCTTTGTTGTCA -3'	
Exons 5-6 forward	5'-AAACTCAAGAGAAGGCTACAAGT -3'	
Exons 7 forward	5'-TGAATGCCAAACTAACAGG	

Koulousios et al.
STROBE Research Checklist

	Item No	
Title and abstract	1	<p>Title [Page 1]: Fabry disease due to D313Y and novel GLA mutations Abstract [Page 3]</p> <p>The study is a polycentric observational cohort study which addresses the question of pathogenicity of specific Fabry disease mutations, using cohort data.</p> <hr/> <p>Abstract [Page 3]: The abstract summarises the objectives, settings and participants, primary and secondary outcome measures, results and conclusions of the study.</p>
Introduction		
Background/rationale	2	<p>Introduction [Page 4]</p> <p>Introduction to the scientific background of Fabry disease and <i>GLA</i> mutations.</p>
Objectives	3	<p>Introduction [Page 5]</p> <p>Aim of the study is to report five novel <i>GLA</i> mutations resulting in FD and provide evidence of pathogenicity of the D313Y mutation regarding which contradictory data have been presented in the literature.</p>
Methods		
Study design	4	<p>Methods - Study design and setting [Page 5]</p> <ul style="list-style-type: none"> - Polycentric observational cohort study, multiple patient cases from various medical units all over Greece (polycentric study). - The study was approved by the institutional review board of the University of Thessaly, Larissa.
Setting	5	<p>Methods - Study design and setting, Clinical assessment [Page 5]</p> <ul style="list-style-type: none"> - Patient cases from multiple medical units in various locations all over Greece. - Family members of the majority of the Greek FD patients, who were diagnosed during the last years. - Pedigree analysis after personal interviews with the selected individuals in their places of residence.
Participants	6	<p>Methods - Study design and setting, Clinical assessment [Page 5]</p> <p><u>Participants of the study fulfilled one of the following eligibility criteria:</u></p> <ul style="list-style-type: none"> i) relatives of a patient with definite FD diagnosis. ii) patient cases with nephrological, cardiac or neurological symptoms suspicious of FD diagnosis from various medical units all over Greece. - Additional genotyping of 145 apparently healthy subjects. - The source of participants selection was their medical records (relation with a FD patient, patients with FD related symptoms), analysed in collaboration with their treating physicians. - Patients follow-up in collaboration with their treating physicians. Revaluation of the clinical status of each selected individual.
Variables	7	<p>Clinical assessment - Laboratory evaluation - Genotyping – [Pages 5-6]</p>

Variables used in the study:

- i) Heart or kidney disease, cerebrovascular events, death at young age and respective causes of death.
- ii) FD related cardiac, renal and neurological signs and symptoms.
- iii) Familiar relationship to a FD patient.
- iv) Enzyme α -Gal A activity in all male subjects.
- v) Lyso-Gb₃ in all subjects.
- vi) Plasma and urine Gb₃ concentration in selected cases.
- vii) Presence of a *GLA* mutation.
- viii) Verification of novel mutations.
- ix) Evaluation of kidney biopsies.

Diagnostic criteria [Page 6]

The diagnosis of FD was posed according to the recently published criteria of the disease [Biegstraaten et al. 2015]

Data sources/ measurement	8	Clinical assessment - Laboratory evaluation - Genotyping – page 5-6 <u>Method of assessment for each variable:</u> <ul style="list-style-type: none">i) Medical history.ii) Physical examination.iii) Pedigree analysis.iv) In DBS by tandem mass spectrometry.v) In DBS by HPLC and tandem spectrometry.vi) Tandem mass spectrometry.vii) Next-generation sequencing.viii) Standard Sanger sequencing using Variant Reporter software v1.1 (Applied Biosystems).ix) Optical and electron microscopy.
Bias	9	Study design and setting [Page 5] <p>Cross examination for the presence of D313Y mutation by genotyping of 145 apparently healthy subjects.</p> Laboratory evaluation [Page 6] <p>Measurement of α-Gal A activity only in male subjects.</p> Genotyping [Page 6] <p>Exclusion of common polymorphisms (UCSC Common SNPs) by bioinformatic analysis using PhyloP, SIFT, Grantham and PolyPhen tools, in comparison to their global (1000 Genomes Global Minor Allele Frequency, ExAC) and European frequency (5000 Exomes European Minor Allele Frequency).</p>
Study size	10	Study design and setting [Page 5] <p>A cohort of 62 subjects from 19 unrelated families was involved in the study. Twenty-five of them were relatives of 9 patients with definite FD diagnosis and 18 were relatives of 10 individuals recruited as carriers of FD traits in whom a GLA mutation was detected.</p>
Quantitative variables	11	Laboratory evaluation [Page 6] <p>In DBS, α-Gal A activity was measured in all male subjects by tandem mass spectrometry, lyso-Gb₃ in all subjects by HPLC and tandem spectrometry as well as</p>

plasma and urine Gb₃ concentration in selected cases by tandem mass spectrometry.

Statistical methods	12	Age of each patient or patient group is presented either as an absolute number or as mean value \pm the Standard Deviation (mean \pm SD).
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Results

Participants	13	<p>Results [Page 7]</p> <p>62 subjects were potentially eligible for participation in the study and were genotyped for a <i>GLA</i> mutation, clinically analysed and under follow-up for a significant period of time.</p> <p>Abstract [page 3]</p> <p>The 62 subjects were selected between 3 categories of patient cases:</p> <ul style="list-style-type: none"> i) Family members of unrelated patients with definite FD diagnosis. ii) Clinically suspected cases. iii) Family members of the previous category.
Descriptive data	14	<p>Abstract [Page 3]</p> <ul style="list-style-type: none"> i) 25 family members of 9 unrelated patients with definite FD diagnosis. ii) Ten clinically suspected cases (nephrological, cardiac or neurological symptoms suspicious of FD diagnosis) from various medical units all over Greece. iii) 18 family members of the previous category. <p>Results [Pages 6-11]</p> <ul style="list-style-type: none"> i) Patients carrying the D313Y mutation: 17 (54\pm14, range 27– 78 years). [page 6] ii) Male patients carrying the D313Y mutation: 7 (61\pm11, range 45– 78 years). [page 6] iii) Female patients carrying the D313Y mutation: 10 (49\pm15, range 27– 70 years). [page 6] iv) Patients carrying novel <i>GLA</i> mutations: 16 members (42\pm19, range 1– 73 years) of 5 unrelated families, all fulfilling the diagnostic criteria of a definite diagnosis of FD. [page 8] v) Patients identified with the novel c.835C>T mutation: 4 members (30\pm24, range 1– 60 years) of a Greek family. Proband of the family: a 31-year-old male with typical FD clinical and laboratory phenotype. [page 8,9] vi) Patients identified with the novel c.280T>A mutation: 4 members (46\pm11, range 30– 56 years) belonging to another Greek family. Proband of the family: a 48-year-old female with typical FD clinical and laboratory phenotype. [page 9] vii) Patients identified with the novel c.924A>C mutation: 3 members (44\pm26, range 17– 69 years) of a Greek family. Proband of the family: a 46-year-old male with typical FD clinical and laboratory phenotype. [page 9,10] viii) Patients identified with the novel c.511G>A mutation: 2 members (37 \pm 4, range 34– 39 years) of an Albanian family living in Greece. Proband of the family: a 39-year-old male with typical FD clinical and laboratory phenotype. [page 10]
Outcome data	15	<p>Results [pages 6-11]</p> <ul style="list-style-type: none"> i) Six (all with definite FD) out of the 62 genotyped subjects were carrying four previously described <i>GLA</i> mutations. [page 6] ii) The D313Y mutation was revealed in 17 individuals, 7 males and 10 females. [page 6] iii) The novel c.835C>T, c.280T>A, c.924A>C and c.511G>A <i>GLA</i> mutations were detected in 13 members of 4 unrelated families.
Main results	16	<p>Results [pages 6-11]</p> <ul style="list-style-type: none"> i) Six (all with definite FD) out of the 62 genotyped subjects were carrying four previously

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- described *GLA* mutations. [page 6]
- ii) The D313Y mutation was revealed in 17 individuals, 7 males and 10 females. [page 6]
- iii) The D313Y mutation was revealed in none of the healthy subjects. [page 6]
- iv) The diagnosis of FD was definitely posed in 5 carriers of the D313Y mutation. [page 6-8]
- v) Two D313Y mutation carriers were patients presenting no FD related signs or symptoms. [page 8]
- vi) Eight D313Y mutation carriers were patients presenting other FD signs mainly neurological that, however, can not document a definite diagnosis of FD. [page 8]
- vii) Two D313Y mutation carriers were apparently healthy. [page 8]
- viii) The diagnosis of FD was definitely posed in all carriers of the novel mutations reported. [page 8-11]

Other analyses	17	i) Bioinformatics analysis of every <i>GLA</i> mutation reported, by (PolyPhen-2) or (SIFT) systems. [page 12-13] ii) Novel mutations verification by standard Sanger sequencing using Variant Reporter software v1.1 (Applied Biosystems). [page 6]
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Discussion

Key results	18	i) Four novel <i>GLA</i> mutations causing classical Fabry Disease are reported. [page 14] ii) Strong evidence that the D313Y mutation could be pathogenic is offered. [page 14]
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Limitations	19	i) The main limitation is the lack of detailed clinical data in older participants. [page 4] ii) Biopsies of affected organs, the gold standard of definite diagnosis, are not available in all cases. [page 4]
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Interpretation	20	Discussion [Pages 11-13] - Five of seventeen carriers of the D313Y mutation proved as suffering definite FD according to the recently published criteria of the disease. The presentation of the disease in our patients indicates that the mutation results in a milder phenotype, with later onset of symptoms. [page 12] - Contradicting results about the pathogenicity of this mutation have been reported in the literature since its first description on 1993. [page 12] - The mutation has been detected in many series of patients presenting signs of FD. However, Niemann et al. describes this variant as non pathogenic, although his two patients were presenting decreased α -gal A activity. Similarly, Oder et al. supports that the D313Y genotype does not lead to severe organ manifestations as seen in genotypes known to be causal for classical FD and Froissart et al. [i] characterizes the mutation as “pseudodeficient allele” implying that it is a sequence variant which encodes an enzyme that is transported to the lysosomes, where it has about 75% of normal enzymatic activity. The D313Y mutation has been also referred as polymorphism. [page 12] - The vast majority of the D313Y patients of the study were presenting with neurological symptoms and signs. [page 12] - Kidney biopsy is significant for a FD diagnosis in patients with renal manifestations of the disease [page 7, 9-11] - The pathogenicity of the four novel mutations reported has been undoubtedly proved as all carriers suffer definite FD. [page 13]
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Generalisability	21	Discussion [Pages 12-13] Conclusion [Page 13-14]
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The study underlines the significance of :

- i) Diagnosis of FD and possible treatment of patients carrying the 5 novel and the D313Y *GLA* mutations.
- ii) Family members genotyping.
- iii) Newborn screening and genetic counselling.
- iv) Avoiding misdiagnoses and crucial delays of diagnosis and treatment of the disease.

The study confirmed the facts that:

- i) Heterozygous females may develop mild to severe FD.
- ii) Misdiagnosis is common in FD.
- iii) Genotype-phenotype correlation does not exist even among the members of the same families.

Other information

Funding 22 **Funding [Page 19]**

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